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Expression of NOS1 and soluble guanylyl cyclase by human kidney epithelial cells: Morphological evidence for an autocrine/paracrine action of nitric oxide

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Expression of NOS1 and soluble guanylyl cyclase by human kidney epithelial cells: Morphological evidence for an autocrine/paracrine action of nitric oxide.

Background. Nitric oxide plays an important role in the kidney through effects on both renal hemodynamics and tubular functions. Tubular epithelial cells are thus a target for nitric oxide. However, as to whether tubular epithelial cells endogeneously produce nitric oxide under physiologic conditions in human kidney is currently unknown. The aim of the present study was to characterize and localize in situ the nitric oxide synthase (NOS) isoforms (NOS1, NOS2, and NOS3) expressed in human normal kidney, and soluble guanylyl cyclase, the well-known target for nitric oxide.

Methods. Five complementary experimental approaches were used: (*1*) detection of NOS reductase activity by nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry, (*2*) immunolocalization of the NOS isoforms (NOS1, NOS2, NOS3), (*3*) immunoblot analysis, (*4*) quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis of NOS mRNA, and (*5*) measurement of NOS activity as the conversion rate of L-[^{14}C]-arginine to L-[^{14}C]-citrulline. In addition, in situ detection of soluble guanylyl cyclase was assessed by immunohistochemistry.

Results. All these techniques led to consistent results showing that epithelial cells of most tubules along the human nephron exhibit functional NOS1, with a corticomedullary gradient observed both at the protein and mRNA levels. Moreover, epithelial cells expressing NOS1 also express soluble guanylyl cyclase, indicating that these cells possess the machinery for autocrine/paracrine effect of nitric oxide.

Conclusion. The present study demonstrates that NOS1 is strongly expressed in most tubules of the human nephron and therefore invites to consider epithelial cells as one of the major source of nitric oxide in the human kidney under physiologic conditions.

Nitric oxide is an inter- and intracellular mediator with diverse physiologic actions, that is synthesized as a by-product originating from the conversion of L-arginine to L-citrulline, in a reaction catalyzed by nitric oxide synthases (NOS) [1]. Three isoforms of NOS exhibiting different distribution and biochemical properties have been identified in mammal tissues: neuronal (nNOS, NOS1), endothelial (eNOS, NOS3), and inducible (iNOS, NOS2) [2, 3]. Constitutive forms of NOS (NOS1 and NOS3) are strictly Ca^{++}-dependent and generate small amounts of nitric oxide, which are generally believed as responsible for modulating the physiologic function of cells. On the contrary, the Ca^{++}-independent iNOS (NOS2) requires several hours for induction, and generates high amounts of nitric oxide. The reactive nitrogen oxide species resulting from these high nitric oxide concentrations can lead to cell injury [4]. However, the distinction between constitutive and inducible NOS has recently appeared not so clear-cut. Indeed, it has been shown that each of the three isoforms can be constitutively expressed in different cell types or be induced by various stimuli. The three NOS isoforms can be expressed in a variety of cells and tissues different from those in which they were initially characterized and cloned [3]. In addition, tissue-specific variants have been described for NOS1 [5–7].

Nitric oxide is well known to have potent influence on kidney function [8] through effects on renin secretion [9], glomerular hemodynamics [10], and medullary blood flow [11]. It was also demonstrated to increase natriuresis without affecting the rate of glomerular filtration. According to several recent studies, this action is due to direct effect on transepithelial ion transport in various

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segments of the nephron. In this respect, nitric oxide was, for instance, shown to inhibit the activity of Na^+,K^+-ATPase and Na^+/H^+ exchanger in proximal tubule [12, 13] and to stimulate K^+ channel in thick ascending limb [14]. An increasing number of investigations in animal models and to a lesser degree in humans indicate that nitric oxide plays also an important role in renal pathophysiology. In support to this view, high amounts of NOS2-derived nitric oxide are involved in the evolution of glomerulonephritis and tubulointerstitial injury in postischemic renal failure [15, 16], or in acute and chronic allograft rejection [17]. NOS1-derived nitric oxide appears to have a key role in the pathogenesis of renal hemodynamic changes in diabetes [18]. Conversely, a decrease in intrarenal production of nitric oxide was reported in animal models of subtotal renal ablation, ureteral obstruction and diabetes, and administration of L-arginine was shown to retard deterioration of kidney function [19–22]. Thus, certainly due to its dual effects as a cytoprotective or deleterious agent, the role of nitric oxide in renal pathology remains unclear and controversial.

Despite the numerous physiologic approaches aiming to establish the role of nitric oxide in renal functions, main studies have been performed in rodents, and human kidney still remains an organ in which the cellular sources of nitric oxide as well as the isoforms of NOS involved have not been fully investigated [16, 17, 23–25].

The aim of the present study was to characterize and localize in situ the NOS isoforms, NOS1, NOS2 and NOS3, expressed in normal human kidney. The expression of NOS was assessed using different approaches, including in situ localization of the nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity of NOS, immunolocalization of NOS isoforms on sections of renal parenchyma, immunoblot analysis, detection of mRNAs, and determination of NOS activity as the conversion rate of L-arginine to L-citrulline. In our hands, this panel of techniques gave consistent results, leading to the conclusion that, besides the expected NOS3 expressing endothelial cells, epithelial cells of most of the renal tubules expressed a functional constitutive NOS of the NOS1 isotype with a corticomedullary gradient observed both at the protein and mRNA levels. In addition, human normal renal kidney did not display NOS2 isoform. Thus, together with endothelial cells, epithelial cells constitute one of the major sources of nitric oxide in the human kidney under physiologic conditions. According to our data, epithelial cells expressing NOS1 also contain soluble guanylyl cyclase, which allows an autocrine/paracrine effect of nitric oxide.

METHODS

Tissue specimens

Normal human renal tissue was collected from 12 patients undergoing nephrectomy for renal tumor. Normal tissue was taken at some distance from the tumor. These samples could be used, according to the French guidelines, since they are considered as residual tissues not relevant to diagnosis. For each case, the cortex, outer medulla, and inner medulla were dissected, snap-frozen, and stored in liquid nitrogen. Normal renal parenchyma was also fixed in 10% formalin and embedded in paraffin. For each case, a histologic examination of the normal kidney was done before performing the experiments. Only samples displaying normal glomeruli and tubules and without any inflammatory infiltrate were selected. A case of inflammatory bowel disease (ulcerative colitis) was used as a positive control for NOS2.

NADPH-diaphorase histochemistry

Five micrometer cryostat sections were fixed for 15 minutes in a freshly made paraformaldehyde solution [4% wt/vol in phosphate-buffered saline (PBS)] and then washed four times in PBS. The NADPH-diaphorase activity of NOS, which is paraformaldehyde-resistant, was demonstrated by enzymatic reduction of nitro blue tetrazolium in the presence of NADPH, as previously described [26]. Briefly, slides were incubated in 1 × PBS containing 1 mg/mL NADPH (Roche, Meylan, France) and 0.4 mg/mL nitro blue tetrazolium (Roche) for 30 minutes at 37°C, washed in PBS, dehydrated, and mounted without any counterstaining. NADPH was omitted in negative controls.

Immunohistochemical staining

Immunohistochemistry was performed on 5 μm formalin-fixed paraffin sections of normal renal parenchyma for staining with the C-term NOS1 and the soluble guanylyl cyclase antibodies, and on 5 μm acetone-fixed cryostat sections for staining with all other antibodies (Table 1). First, endogenous biotin was blocked using a sequential avidin-biotin treatment (Dako Biotin Blocking System, Dako, Trappes, France). Then, immunohistochemistry was performed using a streptavidin-biotin method (Histostain Plus Kit, LAB-SA Detection System, Zymed, Clinisciences, Montrouge, France), according to the manufacturer's instructions. The specificity and conditions of use of the primary antibodies directed to NOS isoforms or soluble guanylyl cyclase are listed in Table 1. Aminoethyl carbazol (AEC) was used as a chromogen. The sections were slightly counterstained with hematoxylin and mounted in glycergel (Dako). Adequate negative controls were performed by omitting the first antibody or using irrelevant antibodies of the same immunoglobulin (Ig) isotype. Immunohistochemical staining was assessed by two independent observers (A.J. and K.R.), including a pathologist (K.R.). The staining was evaluated as (*1*) no staining (−); (*2*) positive staining (+); and (*3*) heterogeneous staining (+*) be-

Table 1. Antibodies used for the detection of nitric oxide synthase (NOS) isoforms and of soluble guanylyl cyclase (sGC) by immunohistochemistry and immunoblot analysis

Protein detected	Type of antibody source	Antigen (amino acids) C- or NH2-terminus	Immunohistochemistry: frozen (F), paraffin (P)/dilution Immunoblot/dilution
NOS1	Monoclonal Transduction Lab	Synthetic human peptide C-terminus (1095–1289)	Immunohistochemistry (F)/1:100 Immunoblot/1:2500
NOS1	Polyclonal Zymed	Rat recombinant protein NH2-terminus (last 200 aa)	Immunohistochemistry (P)/1:200
NOS2	Polyclonal Santa Cruz	Synthetic human peptide C-terminus	Immunohistochemistry (F)/1:500 Immunoblot/1:500
NOS3	Polyclonal Santa Cruz	Synthetic human peptide C-terminus	Immunohistochemistry (F)/1:300
NOS3	Polyclonal Transduction Lab	Synthetic human peptide C-terminus	Immunohistochemistry (F)/1:300 Immunoblot/1:500
sGC	Polyclonal Cayman	Synthetic human peptide from $\alpha 1$ and $\beta 1$ subunits	Immunohistochemistry [(F)/1:800; (P)/1:200]

tween tubules of the same type (distal tubules) or among the various cell types of a given tubule (collecting duct).

Immunoblot analysis

Total proteins were extracted from the 12 specimens of normal kidney (cortex, outer medulla, and inner medulla) using the Tri-Reagent (Euromedex, Mundolsheim, France) [27] according to the manufacturer's instructions. Fifty micrograms of protein were separated by 7.5% SDS-PAGE in reducing conditions and transferred onto nitrocellulose membrane (BioRad, Marnes La Coquette, France). The membranes were treated for 1 hour at room temperature or overnight at 4°C with 1% blocking reagent (Roche), incubated for 1 hour at room temperature with the appropriate NOS antibody diluted in Tris-buffered saline (TBS)/0.5% blocking solution, washed several times in TBS/0.1% Tween, incubated for 45 minutes at room temperature with a rabbit anti-mouse antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Tebu, Le Perray en Yvelines, France), 1:1000 dilution in TBS/0.5% blocking reagent and washed in TBS/Tween. The immunoreactive proteins were then detected by chemiluminescence [BM Chemiluminescence Blotting Substrate (POD), Roche]. Lysates of rat brain for NOS1, ulcerative colitis for NOS2, and human endothelial cells for NOS3 (Transduction Lab, BD Biosciences, Le Pont de Claix, France) served as positive controls.

Quantitative RT-PCR analysis of NOS1

Total RNAs from three specimens of normal kidney (cortex and inner medulla) were isolated using the Tri-Reagent kit (Euromedex) [27]. RNA (5 μg) was denaturated at 72°C for 3 minutes and then reverse transcribed for 60 minutes at 42°C in a 20 μL reaction volume [50 mmol/L Tris-HCl, pH 8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol (DTT) containing 0.5 μg of random hexamers (Promega, Charbonnières, France), desoxynucleoside triphosphate (dNTP) (1 mmol/L each), RNasin (50 units) and RnaseH Moloney-murine leukemia virus (M-MLV) reverse transcriptase (200 units)]. The amplification conditions of the NOS1 and GAPDH templates were optimized for the LightCycler instrument. PCR amplifications were performed using the FastStart™ DNA Master SYBR Green I (Roche Molecular Biochemicals) Real-Time PCR kit. The PCR primers were chosen on separate exons to amplify cDNA but not genomic DNA. The following primers were used: 5′-TCTC CTCCTACTCTGACTCC-3′ (NOS1 sense) and 5′-TTG TGGACATTGGATAGACC-3′ (NOS1 antisense); and 5′-TGAACGGGAAGCTCACTGG-3′ (GAPDH sense) and 5′-TCCACCACCCTGTTGCTGTA 3′(GAPDH antisense). Primers (0.4 μmol/L each) and additional $MgCl_2$ (final concentration 4 mmol/L) were combined with 2 μL FastStart reaction mixture and PCR grade water up to a volume of 18 μL. The glass capillary reaction tubes of the LightCycler were loaded with 18 μL of this master mix and 2 μL of the template (cDNA diluted 1/100). The cycling conditions were as follows: denaturation for 5 minutes at 95°C; amplification for 40 cycles, with denaturation for 0 seconds at 95°C, annealing for 10 seconds at 62°C, and extension for 15 seconds at 72°C. The emitted fluorescence was measured at the end of each cycle. After completion of the cycling process, samples were subjected to a temperature ramp (from 64°C to 95°C) with continuous fluorescence monitoring for melting curve analysis. For each PCR product, a single narrow peak was obtained at the specific melting temperature, indicating specific amplification. A standard curve was generated with serial fourfold dilutions of cDNA (1:10, 1:40, 1:160, and 1:640), prepared from normal kidney RNA. All standards and samples were tested in duplicate and the average value of both duplicates was used for quantification. The amount of NOS1 or GAPDH transcript was calculated from the standard curves using the LightCycler software. It was expressed in relative units, one relative unit corresponding to 1:640 dilution of the standard. For each sample, the ratio between the relative amount of NOS1 and GAPDH was calculated to compensate for variations in quantity or

quality of starting mRNA as well as for differences in reverse transcriptase efficiency.

NOS activity measurement

NOS activity was measured by the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline, in the presence of ornithine (1.5 mmol/L) in order to inhibit arginase activity [28, 29]. Briefly, cortex or inner medulla of three human normal renal kidney were homogenized on ice in a cold buffer containing 50 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.1 mmol/L ethyleneglycol tetraacetate (EGTA), with 5 μg/mL leupeptin, 5 μg/mL aprotinin, and 0.1 mmol/L Pefabloc. Homogeneates were centrifuged at 105,000 *g* during 1 hour and NOS activity was measured in the supernatants and in the pellets solubilized in Tris-HCl buffer. An aliquot (25 μL) was mixed to 100 μL of incubation buffer containing 50 mmol/L Tris-HCl, pH 7.4, 3 mmol/L NADPH, 75 μmol/L $CaCl_2$, 100 nmol/L calmodulin, 4 μmol/L flavin adenine dinucleotide (FAD), 4 μmol/L flavin mononucleotide (FMN), 6 μmol/L BH4, 10 μmol/L L-[^{14}C]-arginine monohydrochloride (313 mCi/mmol, NEN, Paris, France), and incubated 10 mn at 37°C. Stop reaction was promoted by addition of 1 mL of 50 mmol/L HEPES buffer containing 5 mmol/L EDTA, pH 5.5, and 0.5 mL of resin suspension (Dowex 5OWX8-400, Sigma-Aldrich, St. Quentin-Fallavier, France), in order to absorb the L-[^{14}C]-arginine nontransformed into L-[^{14}C]-citrulline. An aliquot of the supernatant was then counted in a liquid scintillation analyzer (Packard Tricarb 2100 TR). Each preparation was tested in duplicate in the presence and absence of NADPH to establish the NADPH-dependent citrulline formation, or in the presence and absence of different inhibitors (100 μmol/L): L-arginine analog *N*-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA), inhibitors of all NOS isoforms, S-methyl-L-thiocitrulline (SMTC) (Cayman, Spibio, Massy, France), a selective NOS1 inhibitor. The constitutive NOS activity was measured in the presence of 1 mmol/L EGTA in a buffer devoid of Ca^{++} and calmodulin. NADPH-dependent activity, NOS activity, or Ca^{++}-dependent activity were determined by substracting total counts from counts remaining in the absence of NADPH, or in the presence of inhibitors or of the Ca^{++}-chelator EGTA. NOS activity was expressed as picomole per milligram protein per minute (pmol/mg/min).

RESULTS

Identification and localization of the different isoforms of NOS (i.e., NOS1, NOS2, NOS3) were assessed in several samples (6 to 12) of normal human renal parenchyma (cortex, outer medulla, and inner medulla), at both protein and mRNA levels using five complementary methods.

In situ detection of NOS reductase activity by NADPH-diaphorase histochemistry

The results of the histochemical detection of NOS activity by NADPH-diaphorase reaction performed on paraformaldehyde-fixed cryostat sections are summarized in Table 2. As shown in Figure 1, NADPH-diaphorase activity was predominant in renal tubules, with a more pronounced staining pattern in cortex and outer medulla than in inner medulla. In the cortex, glomeruli displayed a weak NADPH-diaphorase activity located in capillaries endothelial cells and in some podocytes. Mesangial cells and parietal cells of Bowman's capsule appeared negative. Concerning tubules, epithelial cells of the proximal tubules showed a strong NADPH-diaphorase activity. Those of distal tubules were also positive; however, staining was heterogeneous from one tubule to another. When the juxtaglomerular apparatus was visible, macula densa was strongly positive and endothelial cells of arterioles were faintly labeled. Collecting ducts in the cortex and medulla displayed a moderate NADPH-diaphorase activity. In the outer medulla, NADPH-diaphorase activity predominated in the medullary thick ascending limb of Henle's loops, whereas the thin limbs of Henle's loops appeared faintly stained or even negative. The inner medulla displayed a weak NADPH-diaphorase activity, mainly encountered in collecting ducts and almost absent in thin limbs of Henle's loops. A weak NADPH-diaphorase reaction was also observed in endothelial cells of arterioles, capillaries, and vessels in the cortex and medulla. Some nerve fibers and perivascular nerve bundles were positively marked all along the kidney, especially in the inner medulla and hilar region. All these structures scored positive as early as after 5 to 10 minutes' incubation and no reaction product was observed when NADPH was omitted.

Characterization of the NOS isoforms by immunohistochemistry and immunoblot analysis

The NADPH-diaphorase activity detected in most tubular epithelial cells suggests that these cells are an important source of nitric oxide in the kidney. We then further characterized the NOS isoforms in epithelial and endothelial cells by immunohistochemistry using specific antibodies directed to NOS1, NOS2, and NOS3. In each experiment, negative controls were performed (i.e., by omitting the primary antibody and by using irrelevant antibodies of the same Ig isotype instead of the primary specific antibodies) and staining was always absent.

The "inducible" form of NOS, NOS2, was not detected in normal human kidney. Indeed, it was never found in glomeruli or in the various tubules of the cortex and medulla (Table 2 and Fig. 2A). A positive control for NOS2 was used (i.e., colonic mucosa from a case of ulcerative colitis, known to contain high levels of NOS2

Table 2. Localization of nitric oxide synthase (NOS) and soluble guanylyl cyclase (sGC) in normal human kidney

		Detection of NOS and sGC by immunohistochemistry			
Intrarenal distribution	NADPH-diaphorase activity ($N = 12$)	NOS1 ($N = 12$)	NOS3 ($N = 6$)	NOS2 ($N = 6$)	sGC ($N = 10$)
Glomeruli					
Mesangial cells	−	−	−	−	−
Podocytes	+*	+*	−	−	+*
Parietal cells	−	−	−	−	+*
Cortical tubules					
Macula densa	+	+	−	−	+
Proximal tubules	+	+	−	−	+
Distal tubules	+*	+*	−	−	+
Collecting ducts	+*	+*	−	−	+
Medullary tubules					
Thick ascendant limb HL	+	+	−	−	+
Thin limbs HL	−	−	−	−	+
Collecting ducts	+*	+*	−	−	+
Endothelium	+	−	+	−	+

NADPH is nicotinamide adenine dinucleotide phosphate; HL is Henle's loop.

NADPH-diaphorase reaction and immunohistochemical detection of NOS and sGC were performed on cryostat or paraffin sections of histologically normal human kidney (cortex, outer, and inner medulla for each case), as described in the **Methods** section.

+, positive staining; −, no staining; *, heterogenous staining.

in the epithelium) [30] (Fig. 2B). Immunoblot analysis confirmed the absence of the 130 kD form of NOS2 in normal human kidney (Fig. 2C).

Concerning the "constitutive" isoforms of NOS, we assessed the expression of the neuronal form NOS1 and of the endothelial form NOS3.

Given the strong homologies between the C-terminus part of NOS1 with other proteins having a NADPH reductase activity [3, 26], the expression of NOS1 was assessed by using the monoclonal antibody derived from a peptide mapping at the carboxy terminus of the NOS1 (NOS1-C) and another polyclonal antibody directed to the N-terminus of NOS1 (NOS1-N). In addition, this antibody is suitable for paraffin-embedded sections, thus allowing a better characterization of the tubules and of their cellular components along the nephron. The overall staining pattern was identical with both antibodies and showed that NOS 1 was expressed in tubular epithelial cells all along the nephron (Fig. 3A and Table 2). A higher proportion of tubules scored positive for NOS1 in the cortex and outer medulla than in the inner medulla. In the cortex, proximal tubules and macula densa were strongly stained; distal tubules displayed a heterogenous staining from one tubule to another. Cortical and medullary collecting duct showed a strong reinforced immunoreactivity in the large and round-shaped cells with morphologic characteristics of intercalated cells scattered within the tubule, whereas principal cells, representing the majority of collecting duct cells, displayed a weaker labeling. Concerning Henle's loops, the thick ascending limb was strongly stained while the thin limb was heterogeneously and very weakly stained. In glomeruli, only some podocytes expressed NOS1. Although the staining intensity could vary, it is to be noted that the immunolocalization and distribution of NOS1 were similar from one kidney to another.

To further verify that NOS reactivity detected in renal epithelial cells by immunohistochemistry truly corresponded to NOS1, immunoblots were performed on crude tissue extracts from the main regions of renal parenchyma (cortex, outer medulla, and inner medulla) with the anti-NOS1 antibody. As shown in Figure 3B, which illustrates the results obtained on three representative samples, a band of 155 kD was observed. The intensity of the 155 kD band, although variable from one kidney to another, was far higher in the cortex and outer medulla than in the inner medulla, in which it was in most cases hardly detectable.

NOS3 was expressed in capillary endothelial cells of the glomeruli and in the endothelium of cortical and medullar vessels, as expected. However, NOS3 was never detected in other cell types of the glomeruli and in tubular epithelial cells (Fig. 4A and Table 2). Immunoblot analysis confirmed the expression of the 155 kD form of NOS3 (Fig. 4B).

Altogether, these findings demonstrate that the constitutive NOS of the NOS1 isotype is expressed by epithelial tubular cells in normal human kidney. Expression is moreover heterogeneous, with a gradient of expression (i.e., cortex/outer medulla > inner medulla). NOS3 was present only in endothelial cells of cortical and medullar blood vessels. Moreover, NOS2 was not expressed in normal human renal parenchyma.

Measurement of NOS1 mRNA levels by quantitative RT-PCR analysis

In order to assess whether the corticomedullary gradient of NOS1 expression was a reflection of its mRNA

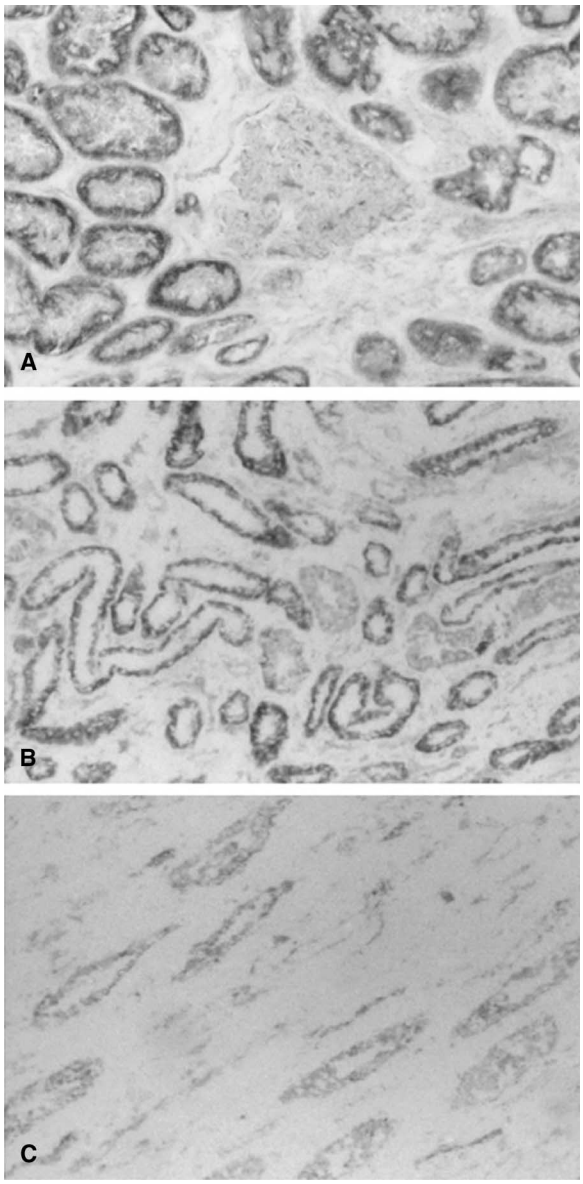


Fig. 1. Localization of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity in normal human renal parenchyma. Assessment of NADPH-diaphorase activity was performed on paraformaldehyde-fixed cryostat sections as described in the **Methods** section. The nitro blue tetrazolium precipitate is highly detectable in tubules, with a more pronounced staining pattern in cortex (*A*) and outer medulla (*B*) than in inner medulla (*C*). A weak NADPH-diaphorase activity is present in capillaries endothelial cells and in some podocytes. Original magnification ×200.

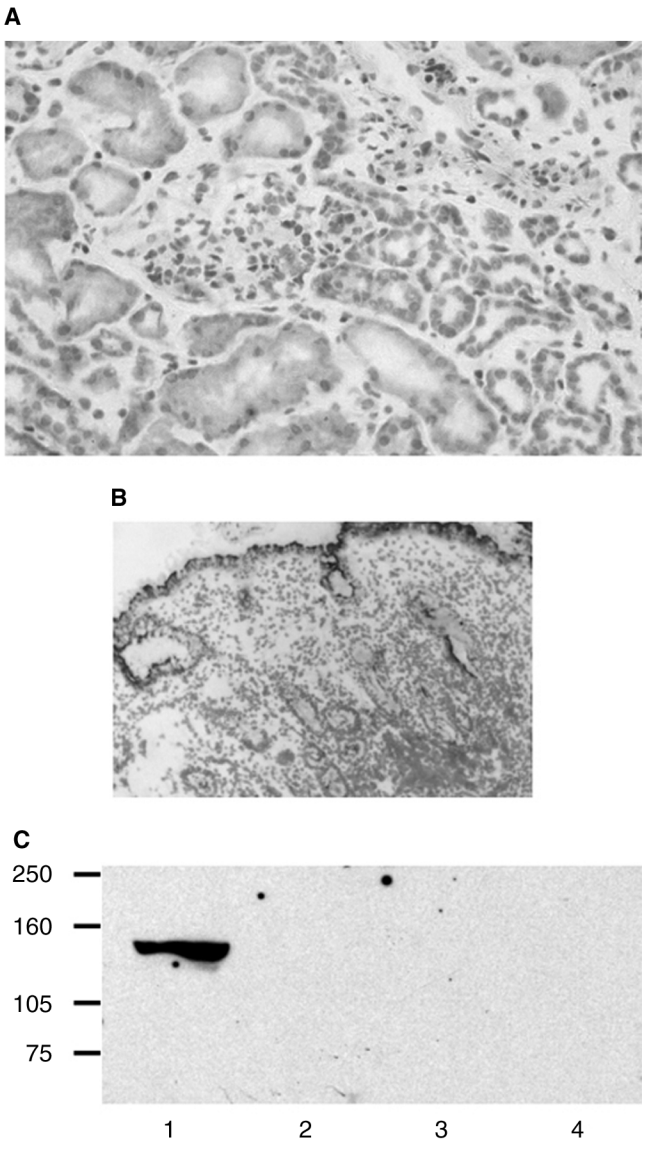


Fig. 2. Analysis of nitric oxide synthase (NOS) NOS2 isoform in normal human kidney. NOS2 was not detected in the cortex of human normal kidney, either by immunohistochemistry on cryostat sections (*A*) (original magnification ×200), or using immunoblot analysis (*C*) (lanes 2, 3, and 4 correspond to three samples of cortex). Immunoblot analysis was performed as described in the **Methods** section. Numbers on the left indicate molecular size of standards in kD. A case of ulcerative colitis, strongly expressing NOS2 in the epithelium, was used as a positive control for immunohistochemistry (*B*) (original magnification ×200) and immunoblot analysis (C) (lane 1).

level, the amount of NOS1 mRNA was measured by quantitative RT-PCR. Analysis of RNA extracted from three kidneys indicated a higher amount of NOS1 amplification product normalized to GAPDH in the cortex than in the inner medulla (Fig. 5). As for immunoblot experiments, the amount of NOS1 amplification product as well as the value of the cortex/medulla ratio varied from one sample to another. The corticomedullary gradient of NOS1 expression was also observed when levels of NOS1 transcripts were normalized to β-actin as a reference gene.

Measurement of a functional NOS activity

NOS activity was measured as the conversion of L-[^{14}C]-arginine into L-[^{14}C]-citrulline, in the cytosolic and pellet fractions of both cortex and inner medulla of three kidney samples. According to the literature, Ca^{++}-dependent NOS activity detected in the cytosolic fraction re-

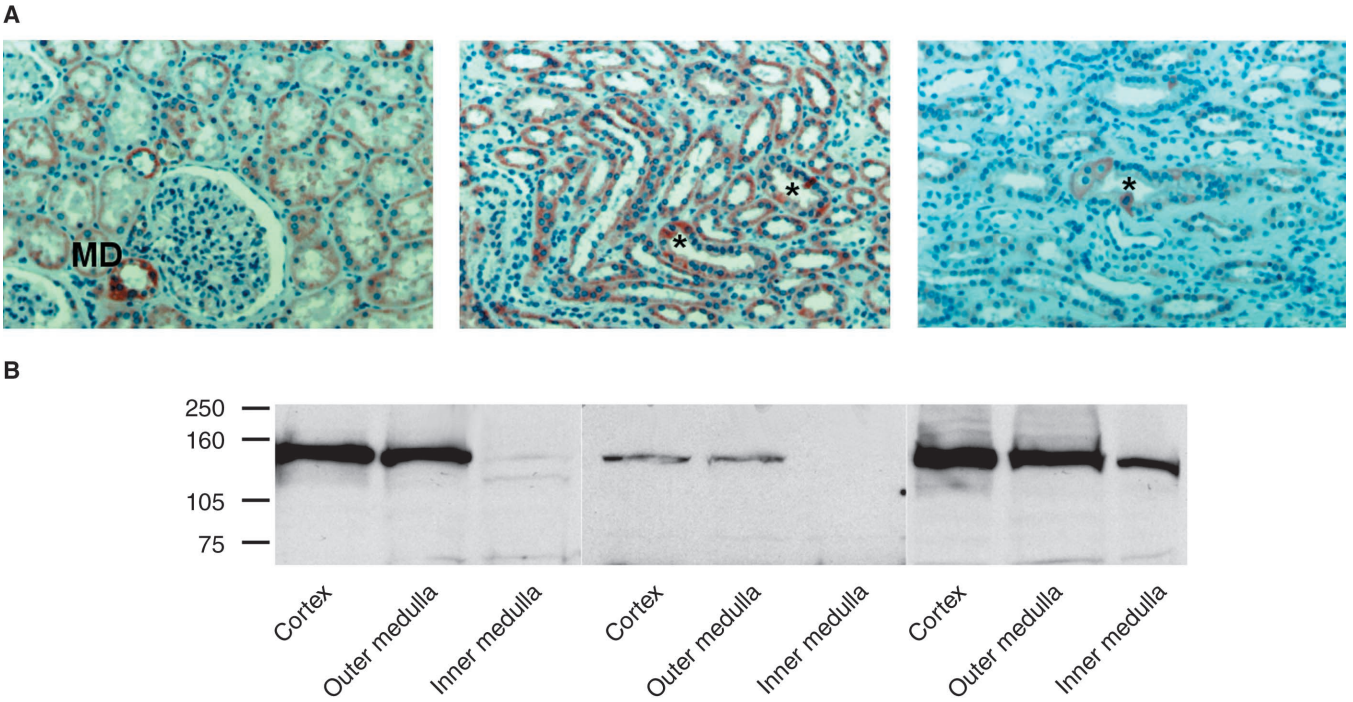
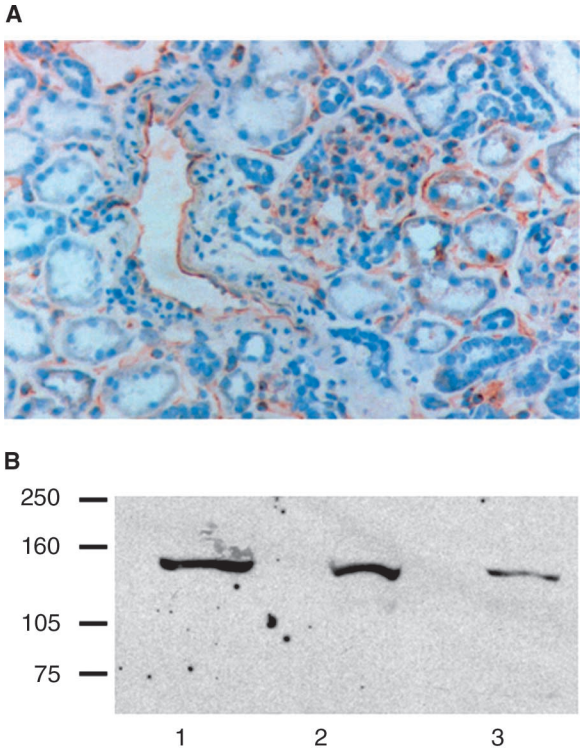


Fig. 3. Localization of nitric oxide synthase (NOS1) isoform in the normal human kidney. (*A*) Immunohistochemical detection of NOS1 in paraffin sections using a polyclonal antibody directed to the N-terminus of NOS1 (left to right: cortex, outer medulla, inner medulla). The cortex showed NOS1 immunoreactivity in the proximal and distal tubules, including the macula densa (MD). In outer medulla, thick ascending limb of Henle's loops strongly expressed NOS1, while thin limbs in the outer and inner medulla did not express NOS1. Medullary collecting ducts expressed NOS1, with a stronger expression in cells with the morphological characteristics of intercalated cells (*) (original magnification ×200). (*B*) Immunoblot analysis of NOS1 expression in the cortex, outer medulla, and inner medulla of three samples of human normal kidney. A cortex/inner medulla gradient of NOS1 protein expression was observed. Immunoblot was performed as described in the **Methods** section. Numbers on the left indicate molecular size of standards in kD.



flects the NOS1 activity, whereas the one detected in the pellet fraction reflects both membrane-bound NOS1 and NOS3 [31, 32]. As in the kidney, other metabolic pathways than NOS can lead to L-[^{14}C]-citrulline formation from L-[^{14}C]-arginine, we measured total L-[^{14}C]-citrulline formation as well as NADPH-, L-NAME-, L-NMMA-, SMTC-dependent L-[^{14}C]-citrulline formation. Figure 6 shows that in the cortex or medulla cytosolic fractions, the NADPH-dependent L-[^{14}C]-citrulline formation and the L-NAME-, L-NMMA-, SMTC-dependent L-[^{14}C]-citrulline formation were very similar. These results strongly suggest that the L-[^{14}C]-citrulline formation measured in these conditions was due to NOS activity. This NOS activity represented 45% and 60% of total L-[^{14}C]-citrulline formation in the cortex and medulla cytosolic

Fig. 4. Analysis of nitric oxide synthase (NOS3) isoform in the human normal kidney. (*A*) Immunohistochemical detection of NOS3 in cryostat sections of cortex. NOS3 was detected only in endothelial cells of glomeruli capillaries, and of the blood vessels present in the cortex. NOS3 was not detected in the tubular epithelial cells (original magnification ×200). (*B*) Immunoblot analysis of NOS3 expression in the cortex of three kidney samples (lanes 1, 2, and 3). Immunoblot was performed as described in the **Methods** section. Numbers on the left indicate molecular size of standards in kD.

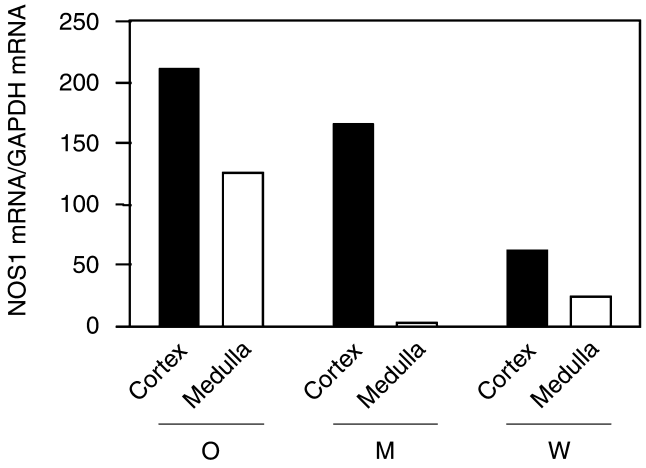


Fig. 5. Quantitative analysis of nitric oxide synthase (NOS1) mRNA levels in normal human renal parenchyma. Total RNAs were extracted from the cortex and the inner medulla of three normal human kidneys (0, M, and W). Five micrograms of each RNA preparation were reverse transcribed and submitted to real-time polymerase chain reaction (PCR) using a Light Cycler apparatus, as described in the **Methods** section. Amounts of NOS1 amplification product were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the three kidneys tested, a higher amount of NOS1 mRNA was observed in the cortex than in the inner medulla.

fractions, respectively, suggesting the existence of other metabolic pathways capable of metabolizing arginine into citrulline. The fact that NADPH-, NAME- or SMTC-dependent NOS activity was abrogated by the Ca^{++}-chelator EGTA indicates the absence of NOS2 in the cortex and medulla. In addition, Figure 6 shows a cortex/inner medulla gradient of NOS activity in the cytosolic fraction, parallell to the gradient of NOS1 expression observed by immunoblot analysis. This gradient of activity was not observed in the pellet fraction, which reflects both NOS3 and membrane-bound NOS1 activities.

Immunolocalization of soluble guanylyl cyclase, a target of nitric oxide

Our finding that renal epithelial cells express a functional NOS strongly suggests that tubules must be an important source of nitric oxide. As soluble guanylyl cyclase is often referred to as the physiologic nitric oxide target, we next determined the immunolocalization of soluble guanylyl cyclase using a polyclonal anti-soluble guanylyl cyclase antibody. Soluble guanylyl cyclase was found to be highly expressed in the various cell types of both cortex and medulla. In the cortex, tubular epithelial cells, which expressed NOS1 were also found to express soluble guanylyl cyclase, with a stronger staining in the proximal tubules (Fig. 4 and Table 2). When visible, macula densa expressed soluble guanylyl cyclase. In glomeruli, soluble guanylyl cyclase antibody labeled most podocytes and some of the parietal cells, but not mesangial cells (Table 2). The various tubules of the outer and inner medulla also expressed soluble guanylyl cyclase, including thin limbs of Henle's loops, which displayed a very faint or even no NOS1 expression (Fig. 7). As already described in other tissues such as lung [33, 34], smooth muscle cells and endothelial cells of blood vessels expressed soluble guanylyl cyclase, and served as an internal positive control.

DISCUSSION

Although the effects of nitric oxide on kidney functions have been extensively studied by using nitric oxide donors and NOS inhibitors, it is unknown yet whether nitric oxide, that is physiologically important for tubule function, is endogenously produced by tubular epithelial cells. Using several complementary approaches allowing to correlate the pattern of enzymatic activity with the immunolocalization of the different NOS isoforms, the present work demonstrates that, besides the expected NOS3 expressing endothelial cells, the epithelial cells of most tubules along the human nephron constitutively expressed a functional NOS1 with a corticomedullary gradient observed both at the protein and mRNA levels.

Evidence for the presence of NOS activity in tubular cells was first provided by examination of NADPH-diaphorase activity on parenchyma sections. There is now consensus that paraformaldehyde-resistant NADPH-diaphorase activity reflects NOS reductase activity [26]. In the only previous study using this methodology on parenchyma sections of human kidney, Bachmann, Bosse, and Mundel [23] found that NOS activity was restricted to cells of the macula densa during the first minutes of incubation (5 to 10 minutes). Activity extended to endothelial cells and to epithelial cells of proximal and distal tubules only after long periods of incubation (up to 1 hour). Although there is no evidence for loss of specificity with time, the tubular and endothelial NOS activities were considered by the authors as a nonspecific signal. In our study, this possibility can be ruled out since NADPH-diaphorase was evident as early as after 5 minutes of incubation in the endothelial cells, macula densa cells and tubular epithelial cells of most of the nephron segments. As shown by the use of several specific antibodies directed to the C- or N-terminus part of NOS isoforms, NADPH-diaphorase was due to NOS1 in macula densa and tubular epithelial cells, and to NOS3 in endothelial cells. These results are consistent with previous work demonstrating that, in the kidney, the expression of NOS3 is restricted to vascular network [8, 23] and that NOS1 is expressed in the macula densa of many species including human [8, 23, 35]. In addition, a main finding of our study is the evidence of a NOS1 expression in tubular epithelial cells, responsible for their NADPH-diaphorase activity. Altogether, our results obtained using histochemistry, immunohistochemistry, immunoblot, and quantitative RT-PCR analy-

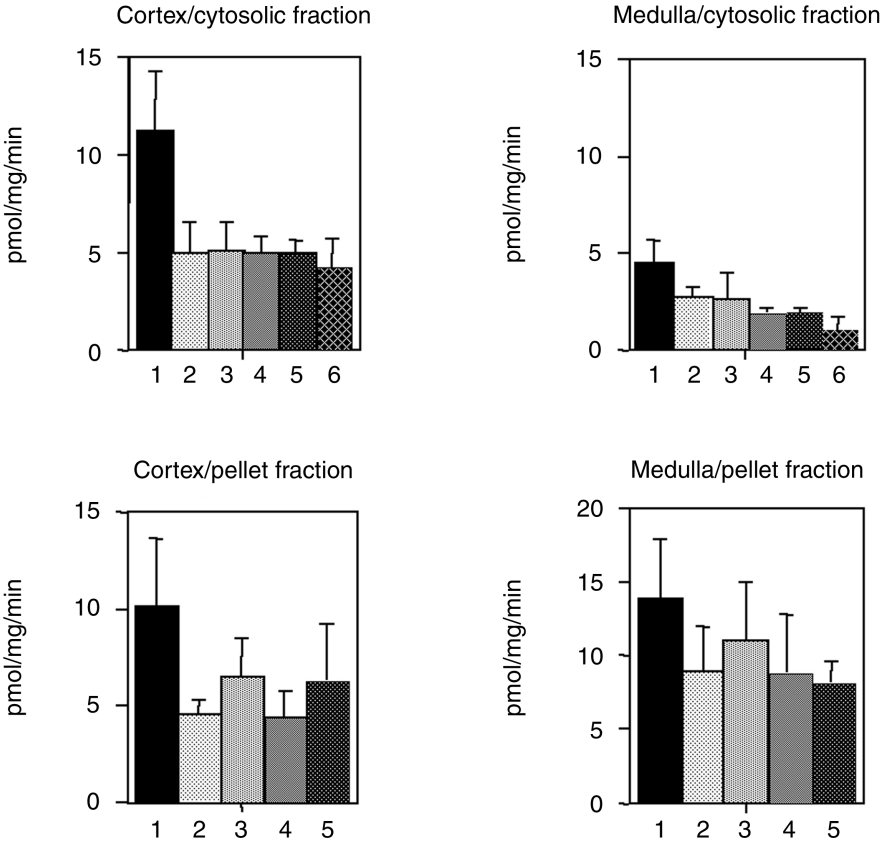


Fig. 6. Measurement of nitric oxide synthase (NOS) activity in the cytosol and pellet fractions of cortex and inner medulla of human normal kidney. Conversion of L-[^{14}C]-arginine into L-[^{14}C]-citrulline was determined as described in the **Methods** section. Results are expressed as picomole/milligram protein/minute (pmol/mg/min). Lane 1 is the total L-[^{14}C]-citrulline formation measured in the absence of inhibitors; lane 2 is the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent L-[^{14}C]-citrulline formation; lane 3 is the L-arginine analog *N*-nitro-L-arginine methyl ester (L-NAME)-dependent L-[^{14}C]-citrulline formation; lane 4 is the SMTC-dependent L-[^{14}C]-citrulline formation; lane 5 is the ethyleneglycol tetraacetate (EGTA)-dependent L-[^{14}C]-citrulline formation; and lane 6 is the N^{G}-monomethyl-L-arginine (L-NMMA)-dependent L-[^{14}C]-citrulline formation.

sis strongly suggest a cortex-inner medulla gradient of NOS1 expression.

Finally, the existence of a constitutive NOS activity was demonstrated by measuring the conversion of L-[^{14}C]-arginine to L-[^{14}C]-citrulline. In fact, our results showed a specific NOS activity in human kidney homogeneates, despite the presence of other metabolic pathways leading to citrulline [29, 36, 37]. These results are in agreement with previous reports on NOS activity in human and rat kidney [8, 17, 24, 25, 38]. Furthermore, in the samples we studied, a constitutive Ca^{++}/calmodulin-dependent NOS activity was detectable in the cytosolic fraction, reflecting NOS1 activity, with a cortex-inner medullar gradient.

The situation in human kidney appears strikingly different from that in rat in several respects. In this species, NADPH-dependent diaphorase activity [39] or Ca^{++}-dependent NOS activity [40] was indeed found several-fold higher in inner medullary than in the cortex. Another major divergence is that epithelial cells were found positive for NOS1 in almost all the segments of the human nephrons. Expression of NOS1 was particularly intense in epithelial cells lining proximal tubules and thick ascendant limbs. Distal tubules and collecting ducts displayed heterogenous staining, whereas thin limbs of Henle were negative. Using RT-PCR on RNA prepared from microdissected rat nephron segments, Terada et al [41] found NOS1 mRNA in inner medullary thin limb and all along the collecting duct. So far the only tubular structure where immunoreactive NOS1 was detected is cortical collecting duct isolated from rats fed with high K^+ diet [42]. However, even in this case, the cellular location of NOS1 appears different from that observed in the present study. Indeed, immunoreactivity was primarily concentrated in cells with morphologic characteristics of principal cells, whereas intercalated cells displayed weak labeling [42]. The reasons for the discrepancies in NOS expression between human and rat remain to be established.

Nitric oxide has been shown to act through a variety of mechanisms, but most of its effects are mediated by a second messager cascade involving the generation of cyclic guanosine monophosphate (cGMP) by soluble guanylyl cyclase [43]. Given also that, with the exception of the inhibition of basolateral K^+ channels in the rat cortical collecting duct, which results from an interaction of nitric oxide with superoxide [44], all the tubular effects of nitric oxide have been primarily ascribed to elevation of cGMP [13, 45, 46], we investigated the immunolocalization of soluble guanylyl cyclase. Soluble guanylyl cy-

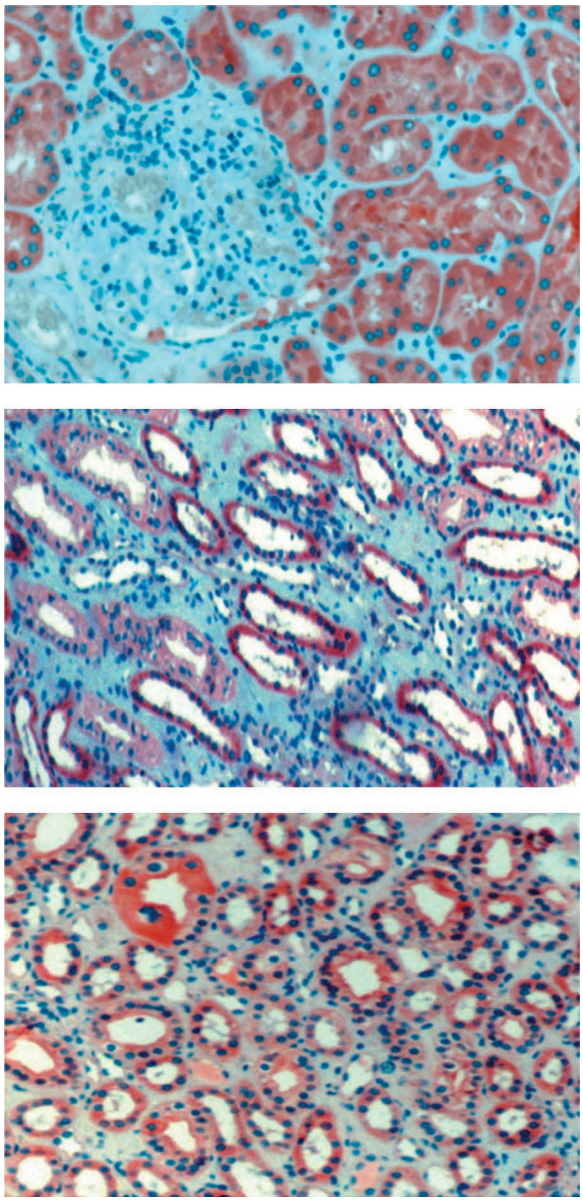


Fig. 7. Immunolocalization of soluble guanylyl cyclase in normal human renal parenchyma. Immunohistochemical detection of soluble guanylyl cyclase on paraffin sections showed a strong staining in proximal and distal tubules of the cortex, as well as in the medullary limbs of Henle's loops and collecting ducts (original magnification ×200).

clase is a heterodimer consisting of one α and one β subunit. Several isoforms of each subunit (α_1, α_2, β_1, β_2) have been cloned. The α_1 β_1 isoenzyme is the major isoform found in animal tissues. However, other isoforms such as α_1 β_2 and α_2 β_1 have also been characterized but their role is poorly understood [43]. In this study, we focus on α_1 β_1 expression in normal human kidney. Interestingly, the study of soluble guanylyl cyclase immunoreactivity indicate a coincident localization of NOS1 and soluble guanylyl cyclase in tubular epithelial cells all along the nephron segment and of NOS3 and soluble guanylyl cyclase in endothelial cells, indicating that both epithelial and endothelial cells possess all the enzymatic machinery necessary for an autocrine/paracrine action of nitric oxide in human kidney. However, soluble guanylyl cyclase is expressed in some cells which do not express NOS1 such as tubular cells of the thin limb of Henle's loop, indicating that these cells can be submitted to a paracrine action of exogeneous nitric oxide originating, for example, from endothelial cells. Additional studies will be necessary to determine the physiologic role of NOS1 in epithelial cells and to establish whether its activity is regulated. Regarding these points, it is noteworthy that inhibition of NOS1 expression by medullar interstitial infusion of antisense oligonucleotide results in hypertension [47] and that inhibitory effect of α_2-adrenoceptor on Cl^- transport are, in isolated thick ascending limbs, mediated by nitric oxide [48].

CONCLUSION

The effects of nitric oxide on kidney function were so far mainly explained by the expression of NOS in endothelial cells and in the juxtaglomerular apparatus. Our demonstration that NOS1 is strongly expressed in epithelial cells of human normal kidney invites to reconsider this scheme as it may bring new insights in the understanding of the tubular effects of nitric oxide on electrolyte transport. In view of the fact that a recent genetic analysis points to the role of NOS1 in chronic renal failure, it is becoming clear that NOS1, in addition to being a player in physiology, might be important in the pathophysiology of renal disease [49].

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